Saccostrea cucullata currently is understood to be indigenous to rocky shores throughout the Indo-Pacific (Lam and Morton, 2006) and along the south and east coasts of Africa (Haupt et al., 2010). The COI and 16S sequences have demonstrated that S. “cucullata” represents a cryptic species complex comprising as many as seven species, often with broadly overlapping geographic distributions (Lam and Morton, 2006; Sekino and Yamashita, 2013). To further complicate the issue, S. “cucullata” is believed to have been introduced to a number of locations where it has become established, including Hawaii (Coles et al., 1999), the Mediterranean Sea, and the Suez Canal (Galil and Zenetos, 2002).

Oysters (Ostreidae) manifest a high degree of phenotypic plasticity, whereby morphology is of limited value for species identification and taxonomy. Among the Ostreidae oysters, the taxonomy of genus Saccostrea is the most problematic because these species have greater morphological plasticity than that of other taxa. Stenzel (1971) believed that Saccostrea cucullata should be regarded as the "monotypic species" or "superspecies" of the genus Saccostrea. Some of the species named in the genera Crassostrea, e.g., C. commercialis and Saccostrea, e.g., S. cucullata, according to Stenzel (1971) and Ahmed (1975), are, in fact, synonymous. This view has relegated (demote) many oysters that grow on rocks and mangroves in the Indo-Pacific area, such as S. commercialis, S. echinata, S. glomerata and S. mordax to either component species of S. cucullata or merely its geographic ecomorphs, or variants. The local rock oyster, S. cucullata, has been misidentified as S. glomerata in his heavy metal investigations of the species (Phillips, 1979). Morris (1985) proposed that the spinose S. kegaki (Torigoe and Inaba, 1981) from Japan (Torigoe and Inaba, 1981) is also included
as a probable synonym of *S. cucullata*. *S. glomerata* and *S. cucullata* both have a flattened growth form extremely similar to that described for *S. glomerata* when individuals of both grow without restriction on a flat surface. Because of this morphology, some Hong Kong *S. cucullata* have been misidentified as *S. glomerata* (Phillips and Yim, 1981). The main morphological character that identifies the genus *Saccostrea* is the presence of chomata in the shell (Stenzel, 1971).

*Saccostrea* oysters mainly inhabit subtropical and tropical areas (Hamaguchi et al., 2014). The Indo-West Pacific rock oyster, *S. cucullata* (Born, 1778), is a common oyster that is widely distributed between the Red Sea and Japan. This species is considered to be a superspecies that contains *S. glomerata* and *S. mordax* (Dinamani, 1976). Only *S. mordax* and *S. kegaki* are clearly distinguished from other *Saccostrea* species. It is not clear whether the remainder of the oysters currently identified as other *Saccostrea* species are actually separate species (Hamaguchi et al., 2014). In Kagoshima Bay, *S. cucullata*-F was originally misidentified as *Crassostrea* species because the chomata was inconspicuous (Sekino and Yamashita, 2013).

The characteristics, i.e., distribution and structure, of mangrove habitats govern the associated biotic communities including oyster population. Other associated parameters of these ecosystems constitute substrate, water movements, pH, water salinity, temperature and abundance of planktonic food. The filtration rate of bivalves depends upon the temperature and speed of water current in ambience.

Temperature is one of the principle variants in benthic communities. Various studies support the idea that sea water temperature affects the metabolic activities such
as growth, reproduction and larval development in most marine invertebrates. For S. cucullata, temperature range of 25-30°C is optimum for embryonic development. The present study recorded the average water temperature in two study locations during the sampling period (August 2011 - July 2012) as 29.7°C ±2.4 and 30.8°C ±2.7 respectively. Oysters are capable of tolerating a wide range of salinity. Salinity is one of the most important environmental factors affecting oyster populations. For S. cucullata, salinities between 25 and 35 ppt are optimum for embryonic development. The present study recorded the range salinity of water ranged from 0.23 ppt to 33.2 ppt and 1.25 ppt to 36.4 ppt in two locations respectively. The species does not reproduce successfully in waters where the pH remained above 9.00. Marked fluctuation was noticed in pH values from 6.8 to 8.8 in the two locations respectively. Dissolved oxygen influences the population and individual growth of oysters and the dissolved oxygen values in the present study fluctuated between 2.5 mg/l and 5.6 mg/l in the two locations respectively. Rainfall helps oysters in relation to food and “fattening” of oysters. In the present study, the total rainfall during sampling period was about 2,445.2 mm, mainly during South-west monsoon in June-September.

A thorough knowledge of the environmental factors affecting feeding, growth, survival and spat fall rate of oysters is essential to initiate successful cultural practices in commercial scale.

The morphological plasticity of Ostreidae oyster species can cause taxonomic confusion. For example, shell morphology has been used as a primary feature to distinguish among species, but it is affected by habitat and environment (Tack et al., 1992; Lam and Morton, 2006, 2009; Wilk and Bieler, 2009). As a result of phenotypic
plasticity, Oysters belonging to Genus *Crassostrea* and *Saccostrea* are difficult to identify by means of their morphology. However, molecular DNA markers are a useful means of discriminating among these species (Varela *et al.*, 2007).

Therefore, Ostreidae oysters should be reclassified using accurate species identification methods. The integration of molecular data with shell morphology can differentiate species, providing new insights into biogeography, invasions, and ecology of this functionally important group (Lohan *et al.*, 2015). Increasingly, molecular markers are being used as an independent data source for species identification, to detect cryptic or unrecognized species, and to clarify persistent taxonomic and biogeographic uncertainty. Molecular markers have been successfully used to differentiate members within the genus *Crassostrea* (e.g., Lam and Morton, 2003; Reece *et al.*, 2008; Cordes *et al.*, 2008; Sekino and Yamashita, 2013) and the genus *Saccostrea* (e.g., Lam and Morton, 2006; Sekino and Yamashita, 2013), and between other closely related oyster genera (e.g., Klinbunga *et al.*, 2005; Liu *et al.*, 2011), as well as aiding in resolving the distributions of other bivalve species. For example, sequence data confirmed the Trans-Atlantic geographic ranges of *C. rhizophorae* and *C. gasar* (Lapégue *et al.*, 2002), as well as the identities and geographic ranges of multiple *Crassostrea* species along the Brazilian coast (de Melo *et al.*, 2010). Furthermore, molecular markers have been used to confirm the introduction of non-indigenous bivalves through anthropogenic activities, such as the previously unrecognized introduction of the Pacific jingle shell, *Anomia peruviana*, to Caribbean waters (Schlöder *et al.*, 2013) and detect potentially non-native species of *Crassostrea* in Brazilian waters (de Melo *et al.*, 2010; Galvão *et al.*, 2013).
By using molecular data, the aim is to genetically characterize the species of *Saccostrea* occurring along the Indian coast, and phylogenetically relate these to other *Saccostrea* species from different parts of the world. Species of *Saccostrea* are common oysters on Indo-Pacific rocky shores. The taxonomic status of *Saccostrea* genus rock oysters in India and along the coastlines of India is unclear and poorly known because of the morphological plasticity among its constituent species. Species of *Saccostrea* from these localities have been identified solely on the basis of shell morphology and anatomy. The present investigation is mainly an attempt to identify *Saccostrea* species using nuclear and mitochondrial genes resolving the taxonomic ambiguity among Indian marine Oyster species. *Crassostrea madrasensis* and *Crassostrea gryphoides* which occur in the same habitat show marked similarity in their external shell morphology (Siddiqui and Ahmed, 2002). Variations in the shape of the oyster shells are likely within the same species from the same area due to overcrowding, orientation, substratum and ecological conditions (Quayle and Newkirk, 1989). Such variations cause great difficulties in the identification of oysters, leading to disagreements among researchers. Recent genetic studies have indicated that identifications of many oyster species based on morphological characters are prone to error (Boudry et al., 1998, Hedgecock et al., 1999, Francis et al., 2000, Klinbunga et al., 2003, Reece et al., 2008).

In this study, RFLP analysis of two nuclear [Internal transcribed spacer (ITS-1) and 18S rDNA] and mitochondrial 16S rDNA gene fragment were used for the identification of *Saccostrea cucullata*. Using Ddel as restriction enzyme (RE) for ITS-1, the bands remained uncut. For 18S the 1100bp was cut by RE into 4 bands 600bp,
275bp, 175bp, 50 bp. For 16S the 400 bp was cut by RE into 2 bands 300bp, 100bp. Using Hinfl as RE for ITS-1 the 500bp yielded 2 bands 450bp, 50bp. For 18S the 1100bp was cut into 3 bands 500bp, 450, 50bp by this RE. For 16S the 400 bp was cut into 2 bands 350bp, 50bp. Using HaeIII as RE for ITS-1 the 500bp was cut into 2 bands 450bp, 50bp. For 18S the 1100bp was cut into 3 bands 500bp, 400bp, 200bp by this RE. For 16S the 400 bp was uncut. These species can be identified with Ddel/Hinfl/HaeIII digestion of ITS-1, 18S rDNA, and 16S rDNA markers. Despite the existence of low frequency in some restriction sites, the unique banding pattern developed in this study can be reliably applied to identify and distinguish *Saccostrea cucullata* from other closely related species. The presence and absence of restricted fragments of each oyster was recorded in a binary matrix. Specific banding pattern could be observed using the 3 restriction enzymes which can be used as a biomarker to identify these species.

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Sequences for nuclear first internal transcribed spacer (ITS-1) region, 18S ribosomal DNA and the mitochondrial 16S ribosomal DNA gene complex obtained after sequencing in this study were deposited to NCBI and GenBank Accession Numbers
KC747110, KC747111 and KC747112 obtained were included in phylogenetic analyses with sequences previously deposited in GenBank. The COI gene has been used in a number of previous oyster molecular phylogenetic analyses, allowing us to directly compare our results with those from the other studies. Use of both a nuclear and a mitochondrial marker allowed identification of a hybrid individual collected from the wild for this study, and sequence information from two independent loci along with new data from oyster species increases confidence in the relationships inferred by results of these analyses.

In the present sequencing analysis study, the homology search using BLASTn showed a maximum 96% identity with 97% query coverage without any error value to ITS gene of *S. commercialis* voucher and a maximum 96% identity with 91% query coverage without any error value to ITS gene of *S. kegaki* voucher. It also showed a maximum 96% and 98% identity with 96% and 50% query coverage without any error value to ITS gene of *S. glomerata* voucher.

For the mitochondrial gene of 16S ribosomal DNA of *Saccostrea cucullata*, the oyster species *Crassostrea madrasensis* is chosen as the outgroup species. The homology search using BLASTn showed a maximum 99% identity with 94% query coverage without any error value to *Saccostrea cucullata* mitochondrial gene for 16S ribosomal RNA voucher and a maximum 94% identity with 96% query coverage without any error value to *Saccostrea echinata* 16S ribosomal RNA gene voucher. It also showed a maximum 94% identity with 96% query coverage without any error value to *Saccostrea palmula* 16S ribosomal RNA gene voucher.
For the mitochondrial gene of 18S ribosomal DNA of *Saccostrea cucullata*, the oyster species *Crassostrea madrasensis* is chosen as the outgroup species. The homology search using BLASTn showed a maximum 98% identity with 98% query coverage without any error value to *Saccostrea kegaki* isolate 18S ribosomal RNA gene voucher and a maximum 97% identity with 41% query coverage without any error value to *Saccostrea glomerata* small subunit ribosomal RNA gene voucher. It also showed a maximum 100% identity with 49% query coverage without any error value to *Crassostrea ariakensis* 18S ribosomal RNA gene voucher and a maximum 98% identity with 45% query coverage without any error value to *Crassostrea gigas* 18S ribosomal RNA gene voucher.

BLAST results of DNA sequences based on nuclear Internal transcribed spacer (ITS-1) and 18S rDNA] and mitochondrial and 16S rDNA] gene sequence datas showed that only mitochondrial 16S rDNA gene sequence data accurately identifies *S. cucullata* (Lam and Morton, 2006). Sc_ITS was found closely related to *S. kegaki*, *S. glomerata* and *S. commercialis* according to the ITS blast report. 18S datasets proved that Sc_18S was closely related to *S. kegaki* and *S. glomerata*. Since there is an absence of 100% identity and query coverage in all the BLAST reports, the identity of these specimens remains unclear and can be confirmed if more specimens are analyzed from different geographic locations.

Lam and Morton (2006) reported that *Saccostrea* species with distinct morphotypes were collected between Japan and Australia. These oysters were reclassified after morphological and mitochondrial-DNA analysis. They discovered 7 groups of *S. cucullata*, *S. kegaki* and *S. glomerata* in one clade, and two groups of
S. mordax in another clade. Total genomic DNA was isolated from the specimens for future accurate species identification by molecular analysis (Hamaguchi et al., 2014) and the Saccostrea oyster specimens were re-identified using DNA barcoding (e.g. Hebert et al., 2003), because the genetic data was used to promote marine fauna biodiversity projects in Japan since 2008. In recent years, genetic information on the oysters that occur in China, Taiwan and Japan has increased (e.g. Liu et al., 2011; Sekino and Yamashita, 2013). Sekino and Yamashita (2013) discovered a new genetic form of S. mordax clade C in Japan that was not reported by Lam and Morton (2006). Molecular phylogenetics revealed first record and invasion of Saccostrea species in the Caribbean (Lohan et al., 2015).

A range of phylogenetic analyses were used to investigate the current taxonomic assignments and evolutionary relationships of the Saccostrea genus. A molecular phylogeny is presented for marine Indian Oyster, Saccostrea cucullata based on two nuclear [Internal transcribed spacer (ITS-1) and 18S rDNA] and two mitochondrial [cytochrome oxidase I (COI) and 16S rDNA] gene sequence data correlated to other Saccostrea species from GenBank (NCBI). S. cucullata were found closely related to S. kegaki, S. glomerata and S. commercialis showing less sequence divergence.

In our study, the Saccostrea oysters were collected from Chorao Island and Nerul Creek of Mandovi - Zuari Estuary in Goa, India. We examined the taxonomic separation between samples by constructing NJ, MP, ML and Bayesian trees using the ITS, 16S and 18S nucleotide sequences. The obtained sequences were aligned using ClustalW v.2.1 (Thompson et al., 1994; gap opening penalty, 10; gap extension penalty, 5). The NJ tree was constructed using MEGA 5.0 (Tamura et al., 2004, 2011) and
Kimura’s two parameter method. From the phylogenetic trees, it is inferred that based on nuclear ITS gene sequence data, *S. cucullata* (Sc_ITS) was closely related to *S. glomerata* and *S. commercialis* with <3% sequence divergence. *Saccostrea kegaki* was the most evolved one. Based on mitochondrial gene of 16S ribosomal RNA gene sequence data all the trees proved that Sc_16S was closely related to *S. cucullata* with <1% sequence divergence. Sc_16S formed distinct clades with *S. cucullata* confirming their specific status and bootstrap support was 100%. Based on mitochondrial gene of 18S ribosomal RNA gene sequence data, Sc_18S was most closely related to *S. kegaki* with high sequence divergence. Sc_18S kept a probabilistic distance of 49.720 from the main clade.

The distance matrix values supported the phylogenies inferred using NJ, MP, ML and Bayesian approaches which produced almost similar, well supported topologies and verified the monophyly of the genus with respect to other *Saccostrea* species. It was proved that the within-species divergence values were greater than 2%. The ITS, 16S and 18S sequences in our study have demonstrated that *S. “cucullata”* is closely related *S. cucullata* is found closely related to *S. kegaki*, *S. glomerata* and *S. commercialis* showing less sequence divergence. Further accurate studies using morphological and molecular techniques are needed to fully resolve the relationships between *Saccostrea* species.

The Phylogenetic trees based on separate analyses of the ITS, 16S and 18S datasets recovered well supported topologies and verified the monophyly of the genus *Saccostrea*. ITS, 16S and 18S datasets showed higher resolutions within intra-species clades and inter-specific evolutionary relationships. *S. cucullata*, *S. glomerata*,
S. *commercialis*, *S. kegaki*, *S. palmula* and *S. echinata* each formed distinct clades, confirming their specific status. ITS datasets showed *S. glomerata*, *S. commercialis* and *S. kegaki* clustered within the same clade and may be closely related to each other or not regarded as a separate species. 16S datasets proved that Sc_16S was *Saccostrea cucullata* since it was clustered in the same clade. 18S datasets proved that *S. kegaki* and *S. glomerata* clustered within the same clade and may be closely related to each other or not regarded as a separate species. The morphologies of *Saccostrea cucullata*, *Saccostrea glomerata*, *Saccostrea kegaki*, and *Saccostrea commercialis*, are so variable and overlapping that it is not always possible to tell them apart by eye. The differentiation of *Saccostrea glomerata* (Sydney Rock Oyster) from *Saccostrea cucullata* is so problematic by the external characteristics at times even for experts that oysters like this are frequently given both names, *S. glomerata cuculata*. In species of *Saccostrea* such as *cucullata*, *glomerata*, and *kegaki* which are all morphologically similar to each other with an oval, deeply cupped left valve and a smaller, relatively flat right valve with slightly plicate, raised margins, even the phylogenetic results using some molecular markers cannot differentiate them. Only mitochondrial 16S rDNA gene sequence data accurately identifies *S. cucullata* (Lam and Morton, 2006).

These results emphasize the need for major systematic revisions within the Ostreidae, namely resolving the species-level relationships within the *Saccostrea* “cucullata” complex as well as a re-examination of the monophyly. Given the functional importance of oysters as ecosystem engineers and their vital roles in many coastal ecological processes (Newell, 2004; Kemp *et al.*, 2005; Coen *et al.*, 2007), including detection of parasites and other problematic microbes (Ford *et al.*, 2009), identifying the
biogeographic distributions for these species is crucial to understanding and managing coastal ecosystems. Finally, this study highlights both the utility and opportunity of combining morphological and molecular tools to resolve identification and biogeography for oysters and other challenging taxonomic groups.

A comprehensive review of morphological studies by adding molecular analysis is needed for species identification. In recent years, some researchers have begun using molecular analysis to reconstruct the taxonomy of the oysters in genus *Saccostrea* that are found in Japan (Hamaguchi *et al.*, 2014).

In our study, in of Mandovi - Zuari Estuary in Goa, we originally misidentified *S. cucullata* and *Crassostrea* species because of the phenotypic plasticity of shells. The molecular data from our study clearly demonstrated that the species we collected is a member of *Saccostrea* genus but assigning these individuals to the correct taxonomical species requires a comprehensive systematic revision of the *S. “cucullata”* complex and is beyond the scope of this analysis. An insight from the molecular phylogenies in this study demonstrates the power of combining molecular with morphological data to confirm the identities of species that are difficult to delineate with morphological characters alone.

A high-resolution molecular diagnostic method would be needed in order to identify non-indigenous *S. cucullata* oysters in the region. DNA barcoding has provided new information that can be used to monitor biodiversity, discover undescribed species, document environmental changes and identify invasive species. In Japan, DNA barcoding has been used to begin taxonomic re-classification of *Saccostrea* oysters (Hamaguchi *et al.*, 2014).